

**REMARKS**

This is in response to the office action dated June 17, 2003. Claims 152, 156, 158, and 167 have been amended. Claim 155 has been canceled, without prejudice or disclaimer. Claim 160 has been allowed. Claims 146-154, 156-173, 175-177, 179-181, 183-185, and 187-189 are pending and at issue.

Claims 152, 156, 158, and 167 have been amended to recite a variant having at least 99% sequence identity to cytochrome P450<sub>cam</sub> from *P. putida* (SEQ ID NO:2). This is supported by the specification at, e.g., page 21, lines 1-15, and by Example 8, pages 51-57.

No new matter has been added by way of this amendment.

**Enablement**

Claims 146-159, 161-173, 175-177, 179-181, 183-185, and 187-189 stand rejected as allegedly not enabled. The Examiner acknowledges that the specification enables a functional cytochrome P450 oxygenase variant having at least 2 to about 10 times the catalytic activity and stability of wild-type cytochrome P450 oxygenase from *P. putida* and comprising mutations at positions 331, 280, and/or 242 of SEQ ID NO:2, but contends that such a variant comprising the above mutations and a 90% sequence identity to SEQ ID NO:2 is not enabled (office action, page 2). The Examiner argues that “[w]hile recombinant and mutagenesis techniques are known, it is not routine in the art to screen for multiple substitutions or multiple modifications, as encompassed by the present claims, and the positions within a protein’s sequence where amino acid modifications can be made with a reasonable expectation of success in obtaining the desired activity/utility are limited in any protein and the result of such modifications is unpredictable”, and goes on to hypothesize that “one skilled in the art would expect any tolerance to modification for a given protein to diminish with each further and additional modification, e.g., multiple substitutions” (page 4-5, bridging paragraph).”

It is respectfully submitted that the pending claims are fully enabled. In brief, the Examiner's arguments relies on the following assumptions: (1) "it is not routine in the art to screen for multiple substitutions or multiple modifications"; (2) "the positions where amino acid substitutions can be made with a reasonable expectation of success are unpredictable", and (3) "tolerance to modification diminishes with each modification." For the reasons discussed below, none of the Examiner's assumptions are fairly based on the description in the specification or the state of the art at the time the present application was filed.

*First assumption: "It is not routine in the art to screen for multiple substitutions or multiple modifications".* Contrary to the Examiner's reasoning, it is routine in the present art, the art of directed evolution, to screen thousands of mutants having one or more substitutions for a particular activity. This was also a routine procedure at the time the present application was filed. Such procedures are, in fact, described in the specification, and are exemplified as follows:

Example 1 (page 28 *et seq.*) describes a whole cell screening procedure for testing the naphtalene hydroxylation activity of cytochrome P450 mutants using the enzyme horse-radish peroxidase (HRP) to "amplify" detection of the oxidation product. As described on page 28, lines 20-21, "there is significant benefit in terms of sensitivity to screening the enzyme mutants for improvements in activity by this method". Example 2 elaborates on the use of a similar technique to screen a large number of cells, describing the use of computer-assisted imaging analysis to scanning of 20,000 colonies of cells. Example 3 describes the use of the same type of screening techniques for alternative oxidation substrates; coumarin and 3-phenylpropionate. Example 4 describes how the sensitivity of the screening technique can be further enhanced by adding a chemiluminiscent substance to the reaction. Examples 5 and 6 describe the use of alternative "amplification enzymes", cytochrome c peroxidase and laccase, in the screening method, Examples 5 and 6, respectively. Example 7 describes another form of "amplified" detection method for detecting dioxygenation of a substrate. Example 9 describes the screening of no less than 55,000 mutant cytochrome P450<sub>cam</sub> clones (page 53, lines 12-13) and about 200,000 mutants (page 53, line 27). Figure 19 describes the analysis results for about 30,000 clones expressing mutant cytochrome



erred in rejecting the applicant's claim to immunoassay methods using a specified generic class of antibodies (IgM). The applicant made a public deposit of a hybridoma cell line that secreted only a specific antibody, but disclosed methods of producing and screening other hybridomas for the desired antibody specificity and class. As summarized by the court (*Id.*):

Enablement is not precluded by the necessity for some experimentation such as routine screening of hybridoma cells that secrete a desired monoclonal antibody from other cells derived from an immunized animal.

In the *In re Wands* case, the applicant's could hardly predict what amino acid sequences would bind to the desired antigen, but performed routine screening to identify antibodies that had the sought-after specificity. This is, again, analogous to the instant claims, where cytochrome P450 variants can be produced and identified from other cells and within a reasonable sequence identity, without prior knowledge as to which positions might or might not lead to retained or improved activity or stability.

*Third assumption: "Tolerance to modification diminishes with each modification.* As for the Examiner's argument that one skilled in the art would expect any tolerance to modification for a given protein to diminish with each further and additional modification, *e.g.*, multiple substitutions, this is in contrast not only to directed evolution techniques but to nature's own evolution. Enzymes evolved from a common ancestor, *e.g.*, orthologs of a certain enzyme present in different types of animals, may frequently exhibit much less than 90% sequence identity albeit retaining a similar function. Likewise, as described above, in directed evolution techniques, several rounds of evolution, where screening is performed in each step to ensure that the desired activity or stability is at least retained or improved, alleviates such concerns, since combinations of mutations resulting in a non-functional enzyme are discarded in the screening process.

As amended, claims 152, 156, 158, and 167 call for 99% sequence identity, and claim 155 has been canceled, without prejudice. Since, as discussed above, the specification enables variants having certain mutations and at least 90% sequence identity to SEQ ID NO:2, variants having 99% sequence identity are clearly also enabled.

As discussed above, the Examiner's assumptions about the enablement of the present invention are not applicable in view of the specification and the state of the art. Moreover, the In re Wands factors referred to by the Examiner on page 3 of the office action are also met by the instant application, as follows:

(1) The quantity of experimentation necessary. The quantity of experimentation, is not excessive and is lessened by the high-throughput screening methods in the Examples coupled with automated sequencing and simple sequence alignment methods. Contrary to the Examiner's contentions, it is routine to screen large numbers of sequences for common properties, such as a specific enzyme activity (P450 activity), particularly if the sequences are derived from a common parent or have a common evolutionary origin.

(2) The amount of direction or guidance presented. The specification provides ample guidance which practitioners can readily follow to practice the invention, including the high-throughput screening methods and sequence alignment methods, as well as assays for and descriptions of cytochrome P450 activity, stability, and specificity.

(3) The presence or absence of working examples. Working examples for preparing, screening for, and identifying the cytochrome P450 variants of the invention are described in the Examples.

(4) The nature of the invention. Practitioners recognize and accept that there is some unpredictability in the field of genetic engineering, and consider screening techniques like those disclosed to be routine and reasonable discriminatory tools. Here, there is specific guidance for using these tools to practice the claimed invention.

(5) and (6) The state of the prior art and the level of skill in the art. These are represented by the enclosed review by Miles et al (Biochim Biophys Acta 2000;1543:383-407), reviewing the P450 family's function, specificity, and structure, and their implications for protein engineering. While this review was published in 2000, the vast majority of the references summarized therein were published before the filing of the present application.

(7) The predictability and unpredictability of the art. While there may be a certain unpredictability in the creation of protein variants using directed evolution, the directed evolution and high-throughput methods described herein enable the creation and screening of a large number of mutants with a reasonable expectation of success.

(8) The breadth of the claims. As amended herewith, the claims describe and distinctly claim the cytochrome P450 variants of the invention, which have mutations at one or more specific sites and a certain amino acid sequence identity to SEQ ID NO:2. The claims are therefore not unduly broad.

Thus, for all of the above reasons, reconsideration and withdrawal of this rejection is respectfully requested.

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In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to pass this application to issue.

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Respectfully submitted,

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